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Date of Deposit: December 28, 2001
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APPLICATION FOR UNITED STATES LETTERS PATENT

S P E C I F I C A T I O N

TO ALL WHOM IT MAY CONCERN:

Be it known that I, Kunal Saha a citizen of the United States of America, residing at 3937 Kul Circle South, Hilliard, 43026, in the State of Ohio have invented new and useful METHODS AND MATERIALS RELATING TO CD8-TROPIC HIV-1, of which the following is a specification.

METHODS AND MATERIALS RELATING TO CD8-TROPIC HIV-1

Scientific work relating to the present invention was supported by two grants from the National Institutes of Health AI42715 and AI 44974. The United States government may have certain rights in the invention.

Related Applications

The present application claims priority benefit of United States Provisional Application No. 60/258,472 filed December 28, 2000 which is herein incorporated by reference in its entirety.

Field of the Invention

The present invention relates to human immunodeficiency viruses 1 (HIV-1) that infect CD8-positive T lymphocytes using CD8 as a receptor, to detection methods for the viruses and to prophylactic and therapeutic methods for infection by the viruses.

Background

HIV-1 is considered to be the causative agent of Acquired Immunodeficiency Syndrome (AIDS) in the United States. HIV-1 infection is characterized by an asymptomatic period between infection with the virus and the development of AIDS. The rate of progression to AIDS varies among infected individuals. AIDS involves the infection and eventual depletion of a particular type of cell of the immune system, cells that have a protein named CD4 protein on their surface (CD4-positive cells). Helper T cells and monocytes/macrophages are CD4-positive cells.

The process by which HIV-1 infects human cells involves interaction of proteins on the surface of the virus with proteins on the surface of the cells. The common understanding is that the first step in HIV infection is the binding of HIV-1 glycoprotein (gp) 120 to cellular CD4 protein. The viral gp120 then changes conformation or shape and binds to yet other cell surface proteins, such as CCR5 or CXCR4 proteins, allowing subsequent fusion of the virus with the cell. CD4 has thus been described as the primary receptor for HIV-1 and the other cell surface proteins as coreceptors for HIV-1.

More recently, there have been reports of certain HIV-1 viruses that can infect cells without binding to CD4. In Dumonceaux *et al., J. Virol., 72*(1): 512-519 (1998), the authors describe an HIV-1 strain that is capable of infecting cells that do not have CD4 on their surface. They observe that the amino acid sequence of the gp120 of that HIV-1 strain had changed in a way that altered the gp120 conformation and enabled it to bind to the coreceptor CXCR4 without first binding to CD4. The HIV-1 strain described in the article is a long-term laboratory culture rather than a primary isolate (a virus sample obtained directly from an infected individual). In Kolchinsky *et al., J. Virol., 73*(10): 8120-8126 (1999), the authors report adapting a HIV-1 virus to infect canine cells lacking CD4 on their surface. The adapted virus was able to bind CCR5 without first binding CD4. They also attribute this CD4-independent infection to changes in the amino acid sequence and conformation of the gp120 of their virus.

While the foregoing reports involved HIV-1 sequence changes in the laboratory, HIV-1 is known to undergo sequence changes in infected individuals. Termed viral evolution, viral sequence changes are believed to be one of the mechanisms by which HIV-1 evades the human immune response. For example, HIV-1 sequence changes that allow it to utilize different coreceptors and thereby infect different types of cells have been described in Shankarappa *et al., J. Virol., 73*(12): 10489-10502 (1999) and Hoffman *et al., Proc. Natl. Acad. Sci. USA, 95*:11360-11365 (1998).

Another type of immune system cell, cytotoxic T lymphocytes that express the CD8 protein on their surface (CD8-positive cells), play an important protective role against HIV-1. CD8-positive T cells kill HIV-infected cells and release antiviral factors which are thought to inhibit the replication of HIV-1 and prevent progression to AIDS. Previous studies have demonstrated that HIV-1 can occasionally infect CD8-positive T cells, but infection of the CD8-positive cells was attributed to binding of the virus to CD4 receptors on the cells. See, Flamand *et al., Proc. Natl. Acad. Sci. USA, 95*: 3111-3116 (1998) and Kitchen *et al., J. Virol., 72*: 9054-9060 (1998). The authors of Kaneko *et al., J. Virol., 71*(11): 8918-8922 (1997) had previously reported that HIV-2 gp105, but not HIV-1 gp120, can bind to the CD8 protein on human cells. HIV-2 is another human immunodeficiency virus that causes AIDS and is prevalent in

developing countries. It is more closely related to simian immunodeficiency virus (SIV) than to HIV-1 but also uses CD4 as its primary receptor for infection. Declines in both the number of CD8-positive T cells and specific anti-HIV cytotoxic activity are associated with the onset of AIDS.

There thus remains a need in art for a more complete understanding of the type of human cells infected by HIV-1 as well as the mechanism by which infection occurs to allow the development of vaccines to prevent, and drugs to treat, HIV-1 infection.

Summary of the Invention

The present invention provides methods and materials for detecting, preventing and treating HIV-1 infection that relate to the previously unrecognized ability of HIV-1 to infect human CD8-positive cells using CD8 as a receptor. HIV-1 viruses that infect CD8-positive cells using CD8 as a receptor are defined herein as CD8-tropic HIV-1. CD8-tropic HIV-1 may or may not utilize one or more other receptors/coreceptors to infect CD8-positive cells.

The CD8-positive cells infected by CD8-tropic HIV-1 may be CD4-negative or CD4-positive. CD8-tropic HIV-1 that retain the ability to infect CD4-positive, CD8-negative cells are defined herein as dual (CD4/CD8)-tropic HIV-1.

Therefore in one aspect the invention provides CD8-tropic HIV-1. Exemplary CD8-tropic HIV-1 are named AD3.v6 and AD3.v22. AD3.v6 and AD3.v22 are dual tropic viruses which were isolated from a single individual. Additional examples of CD8-tropic viruses are named 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8 which are also dual tropic viruses isolated from seven different individuals. Other CD8-tropic HIV-1 can be isolated from an infected individual as follows. Viruses from a patient (as routinely isolated from viral supernatants or plasma) are first used to infect a purified (CD4-positive cell-depleted) population of CD8-positive cells. The infected population is then further purified by sorting the cells by FACS after labeling with anti-CD8 and anti-CD4 antibodies and selecting CD8-positive, CD4-negative cells. The sorted cells are then confirmed to be CD4-negative by testing for the absence of mRNA for CD4.

The sorted cells which are highly purified CD8-positive cells are then cultured for growth by methods standard in the art of CD8-tropic HIV-1.

Also provided by the invention are isolated gp120 polypeptides of CD8-tropic HIV-1. The sequences of illustrative gp120 polypeptides, those of AD3.v6 and AD3.v22, are set out in Fig. 3 (SEQ ID NO: 2 and 4, respectively) and those of 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8 are set out in Fig. 6 (SEQ ID NOS: 10, 12, 14, 16, 18, 20 and 22, respectively). The polypeptides may be full length gp120 polypeptides as well as gp120 polypeptide fragments that comprise an antigenic epitope unique to a CD8-tropic HIV-1 or comprise a binding site for CD8 unique to a CD8-tropic HIV-1. These gp120 fragments may result from truncations at the amino terminus (with or without a leader sequence), truncations at the carboxy terminus, and/or deletions internal to the polypeptide. The isolated gp120 polypeptides of the CD8-tropic viruses of the present invention include regions within the polypeptide which are contemplated to confer the ability to utilize CD8 as a receptor. Therefore, fragments which include one or more of these regions are provided by the invention. The CD8-tropic HIV-1 gp120 polypeptides may be a chimeric polypeptide comprising amino acids from a different CD8-tropic HIV-1 or comprising amino acids other than CD8-tropic HIV-1 amino acids. The activity of the viruses comprising gp120 polypeptides of the invention is evaluated by routine screening assays. Examples of screening assays are described herein such as assays detecting viral infection of CD8-positive/CD4-negative cells (*e.g.*, KRCD8) and the ability to form syncitia in CD8-positive/CD4-negative cells.

The invention also provides for CD8-tropic gp120 polypeptides with one or more amino acid substitutions within the V1-V2, C2 and V4 loops. Substitutions within these regions are contemplated to impart CD8-tropism to CD8-tropic viruses. Specifically contemplated are CD8-tropic gp120 polypeptides which have, for example, an isoleucine residue at position 270; a aspartic acid or glutamic acid residue at position 177: a serine residue at position 209; a glutamic acid residue at position 352; and/or a glutamic acid residue at position 442. Also contemplated are CD8-tropic gp41 polypeptides which have, for example, an isoleucine at position 693; a gluatamic

acid residue at position 724; an alanine residue at position 779; and/or an insertion of HSSLKGL (SEQ ID NO: 27) within the transmembrane domain.

The invention also provides for CD8-tropic gp120 polypeptides with one or more conservative amino acid substitutions that do not affect the cellular tropism of the virus. Alternatively, the CD8-tropic gp120 polypeptides of the invention are contemplated to have conservative amino acids substitutions which may not confer CD8-tropism but are associated with or enhance CD8-tropism. The term "conservative amino acid substitution" refers to a substitution of a native amino acid residue with a nonnative residue, including naturally occurring and nonnaturally occurring amino acids, such that there is little or no effect on the polarity or charge of the amino acid residue at that position. For example, a conservative substitution results from the replacement of a non-polar residue in a polypeptide with any other non-polar residue. Further, any native residue in the polypeptide may also be substituted with alanine, as has been previously described for "alanine scanning mutagenesis". Naturally occurring amino acids are characterized based on their side chains as follows: basic: arginine, lysine, histidine; acidic: glutamic acid, aspartic acid; uncharged polar: glutamine, asparagine, serine, threonine, tyrosine; and non-polar: phenylalanine, tryptophan, cysteine, glycine, alanine, valine, proline, methionine, leucine, norleucine, isoleucine General rules for amino acid substitutions are set forth in Table 1 below.

Table 1

Amino Acid Substitutions

Original Residues	Exemplary Substitutions	Preferred Substitutions
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln	Gln
Asp	Glu	Glu
Cys	Ser, Ala	Ser
Gln	Asn	Asn
Glu	Asp	Asn
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe,	Leu
Leu	Norleucine, Ile, Val, Met,	Leu
Lys	Arg, 1,4 Diaminobutyric	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Arg
Pro	Ala	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala,	Leu

The invention provides isolated polynucleotides encoding CD8-tropic HIV-1 gp120 polypeptides of the invention. The polynucleotides may comprise DNA or RNA. The sequences of illustrative gp120 polynucleotides, those of AD3.v6, AD3.v22, 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8 , respectively, are set out in Fig. 1, 2 and 7-13 as SEQ ID NOS: 1, 3, 9, 11, 13, 15, 17, 19 and 21. The polynucleotides set out in the figures herein consist of the nucleotide sequence encoding the full length envelope polypeptides (gp120 and gp41). Other CD8-tropic HIV-1 gp120 polynucleotides may

be identified and/or isolated by stringent hybridization with AD3.v6 or AD3.v22 polynucleotides or by PCR using primers based on those polynucleotides.

Hybridization stringency is principally determined by temperature, ionic strength, and the concentration of denaturing agents such as formamide. Examples of stringent conditions for hybridization and washing are 0.015 M sodium chloride, 0.0015 M sodium citrate at 65-68°C or 0.015 M sodium chloride, 0.0015M sodium citrate, and 50% formamide at 42°C. See Sambrook *et al., Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, (Cold Spring Harbor, N.Y. 1989). More stringent conditions (such as higher temperature, lower ionic strength, higher formamide, or other denaturing agent) may also be used, however, the rate of hybridization will be affected. Other agents may be included in the hybridization and washing buffers for the purpose of reducing non-specific and/or background hybridization. Examples are 0.1% bovine serum albumin, 0.1% polyvinyl-pyrrolidone, 0.1% sodium pyrophosphate, 0.1% sodium dodecylsulfate, $NaDodSO_4$, (SDS), ficoll, Denhardt's solution, sonicated salmon sperm DNA (or other non-complementary DNA), and dextran sulfate, although other suitable agents can also be used. The concentration and types of these additives can be changed without substantially affecting the stringency of the hybridization conditions. Hybridization experiments are usually carried out at pH 6.8-7.4, however, at typical ionic strength conditions, the rate of hybridization is nearly independent of pH. *See* Anderson *et al.*, *Nucleic Acid Hybridisation: A Practical Approach*, Ch. 4, IRL Press Limited (Oxford, England). Hybridization conditions can be adjusted by one skilled in the art in order to accommodate these variables and allow DNAs of different sequence relatedness to form hybrids.

Antisense polynucleotides complementary to the polynucleotides encoding the CD8-tropic HIV-1 gp120 polypeptides are also provided.

The invention contemplates that polynucleotides of the invention may be inserted in a vector for amplification or expression. For expression, the polynucleotides are operatively linked to appropriate expression control sequence such as a promoter and polyadenylation signal sequences. Further provided are prokaryotic and eukaryotic cells comprising polynucleotides of the invention.

Exemplary prokaryotic hosts include bacteria such as *E. coli*, Bacillus, Streptomyces, Pseudomonas, Salmonella and Serratia. Eukaryotic host cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO), human embryonic kidney cells (HEK), 293 or 293T cells, 3T3 cells, mouse neuroblastoma N2A cells, HeLa cells, mouse L-929 cells, BHK or HaK hamster cell lines; insect cell lines such as SF-5 or Hi-5, or yeast cells such as Saccharomyces, Pichia, Candida, Hansenula, and Torulopsis.

The term isolated is used herein to refer to a substance removed from, and essentially free of, the other components of the environment in which it naturally exists. For example, a polypeptide is separated from other cellular proteins or a DNA is separated from other DNA flanking it in a genome in which it naturally occurs.

The invention provides antibodies which bind to antigenic epitopes unique to (*i.e.*, are specific for) a CD8-tropic HIV-1. Also provided are antibodies which bind to antigenic epitopes common among multiple CD8-tropic HIV-1 but unique with respect to any other antigenic epitopes. The antibodies may be polyclonal antibodies, monoclonal antibodies, antibody fragments which retain their ability to bind their unique epitope (for example, Fv, Fab and F(ab)2 fragments), single chain antibodies and human or humanized antibodies. Antibodies may be generated by techniques standard in the art.

The invention contemplates methods of eliciting an immune response to a CD8-tropic HIV-1 in an individual wherein the antibodies elicited block binding of CD8-tropic HIV-1 to CD8. In one embodiment, the methods comprise a step of administering an immunogenic dose of a composition comprising a CD8-tropic HIV-1 gp120 polypeptide of the invention. In another embodiment, the methods comprise administering an immunogenic dose of a composition comprising a cell expressing a CD8-tropic HIV-1 gp120 polypeptide of the invention. In yet another embodiment, the methods comprise administering an immunogenic dose of a composition comprising a polynucleotide encoding a CD8-tropic HIV-1 gp120 polypeptide of the invention. The polynucleotide may be a naked polynucleotide not associated with any other nucleic acid or may be in a vector such as a plasmid or viral vector (*e.g.*, adeno-associated virus vector or adenovirus vector). Administration of the compositions

may be by routes standard in the art, for example, parenteral, intravenous, oral, buccal, nasal, pulmonary, rectal, or vaginal. The methods may be used in combination in a single individual. The methods may be used prior or subsequent to infection of an individual with HIV-1.

The invention correspondingly provides compositions suitable for eliciting an immune response to CD8-tropic HIV-1, wherein the antibodies elicited block binding of CD8-tropic HIV-1 to CD8. The compositions comprise CD8-tropic HIV-1 gp120 polypeptides of the invention, cells expressing the polypeptide, or polynucleotides encoding the polypeptides. The compositions may also comprise other ingredients such as carriers and adjuvants. An immunogenic dose of a composition of the invention is one that generates, after administration, a detectable humoral and/or cellular immune response in comparison to the immune response detectable before administration or in comparison to a standard immune response before adminstration. The invention contemplates that the immune response resulting from the methods may be protective and/or therapeutic.

Also provided by the invention are methods for detecting CD8-tropic HIV-1. In one embodiment, the methods comprise detecting CD8-tropic HIV-1 gp120 polynucleotide of the invention in a sample using primers or probes that specifically bind to the polynucleotide. Detection of the polynucleotide may be accomplished by numerous techniques routine in the art involving, for example, hybridization and PCR. In another embodiment, the methods comprise detecting CD8-tropic HIV-1 gp120 polypeptide of the invention in a sample using antibodies of the invention. Detection of the polypeptide may also be accomplished by numerous techniques routine in the art such as by ELISA or Western blotting.

The invention includes methods of blocking binding of CD8-tropic HIV-1 to CD8-positive cells in an individual in need thereof. The methods comprise administering antibodies or polypeptides of the invention that block binding of CD8-tropic HIV-1 to CD8. Alternatively, administration of one or more small molecules that block binding of CD8-tropic HIV-1 to CD8 is contemplated. As still another embodiment, the methods comprise administration of anti-CD8 antibodies. *In vitro*

assays may be used to demonstrate the ability of an antibody, polypeptide or small molecule of the invention to block binding of CD8-tropic HIV-1 to CD8.

Pharmaceutical compositions comprising antibodies of the invention, polypeptides of the invention and/or small molecules of the invention that block binding of CD8-tropic HIV-1 to CD8 are provided. The pharmaceutical compositions may consist of one of the foregoing active ingredients alone, may comprise combinations of the foregoing active ingredients or may comprise additional active ingredients used to treat HIV-1 infection, for example, anti-CD8 antibodies, IL-2, protease inhibitors, reverse transcriptase inhibitors, interferons, AZT and cytokines. The pharmaceutical compositions may comprise one or more additional ingredients such as pharmaceutically effective carriers. Dosage and frequency of the adminstration of the pharmaceutical compositions are determined by standard techniques and depend, for example, on the weight and age of the individual, the route of administration, and the severity of symptoms. Administration of the pharmaceutical compositions may be by routes standard in the art, for example, parenteral, intravenous, oral, buccal, nasal, pulmonary, rectal, or vaginal.

Brief Description of the Drawing

Numerous other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof, reference being made to the drawing wherein:

Figure 1 is a DNA sequence encoding the gp120 envelope polypeptide of AD3.v6 (SEQ ID NO: 1);

Figure 2 is a DNA sequence encoding the gp120 envelope polypeptide of AD3.v22 (SEQ ID NO: 3);

Figure 3 is a DNA sequence encoding the gp120 envelope polypeptide of HXB2 (SEQ ID NO: 5);

Figure 4A-4C is an alignment of the gp120 envelope polypeptide sequences of AD3.v6 (SEQ ID NO: 2) and AD3.v22 (SEQ ID NO: 4) with the gp120 envelope polypeptide sequences of HXB2 (SEQ ID NO: 6), a prototype CD4-tropic HIV-1 virus, and of WEAU1.6 (SEQ ID NO: 8), the closest matched isolate;

Figure 5 is a DNA sequence encoding the gp120 envelope polypeptide of WEAU1.6 (SEQ ID NO: 7);

Figure 6A-6D sets out the envelope polypeptide sequences of 92UG046-T8 (SEQ ID NO: 10), 93UG086-T8 (SEQ ID NO: 12), 92US077-T8 (SEQ ID NO: 14), 93US143-T8 (SEQ ID NO: 16), 96USHIPS4-T8 (SEQ ID NO: 18, 96USHIPS9-T8(SEQ ID NO: 20), 96USSN20-T8 (SEQ ID NO: 22), HXB2 (SEQ ID NO: 6; a prototype CD4-tropic HIV-1 virus) and AD3.v6 (SEQ ID NO: 2); wherein the sequences displayed consist of the full length envelope polypeptides (gp120 and gp41) and the residue where the gp120 polypeptide ends is clearly indicated in the figure;

Figure 7 is a DNA sequence encoding the gp120 envelope polypeptide of 92UG046-T8 (SEQ ID NO: 9);

Figure 8 is a DNA sequence encoding the gp120 envelope polypeptide of 93UG086-T8 (SEQ ID NO: 11);

Figure 9 is a DNA sequence encoding the gp120 envelope polypeptide of 92US077-T8 (SEQ ID NO: 13);

Figure10 is a DNA sequence encoding the gp120 envelope polypeptide of 93US143-T8 (SEQ ID NO: 15);

Figure 11 is a DNA sequence encoding the gp120 envelope polypeptide of 96USHIPS4-T8 (SEQ ID NO: 17);

Figure 12 is a DNA sequence encoding the gp120 envelope polypeptide of 96USHIPS9-T8 (SEQ ID NO: 19); and

Figure 8 is a DNA sequence encoding the gp120 envelope polypeptide of 96USSN20-T8 (SEQ ID NO: 21).

Detailed Description of the Invention

The present invention is illustrated by the following examples relating to CD8-tropic HIV-1. Example 1 describes experiments demonstrating the ability of HIV-1 viruses AD3.v6 and AD3.v22 to infect CD8-positive peripheral blood lymphocytes. In Examples 2 and 3, respectively, AD3.v6 and AD3.v22 are shown to use CD8 receptors to infect a CD8-positive, CD4-negative T cell line and to infect CD8-transfected HeLa and COS cell lines. Example 4 describes the ability of anti-CD8

antibodies to inhibit replication of AD3.v6 and Ad3.v22 in primary CD8-positive cells. An analysis of the sequences of the AD3.v6 and AD3.v22 gp120 envelope sequences is presented in Example 5. Examples 6-10 describe the isolation and characterization of additional CD8-tropic quasispecies from various patients. Finally, implications of the CD8-tropism of viruses of the invention are discussed in Example 11.

Example 1

HIV-1 viruses AD3.v6 and AD3.v22 were isolated from a single patient and were demonstrated to infect CD8-positive peripheral blood lymphocytes (PBL).

In one type of experiment, PHA-activated total PBL and purified populations of CD4-positive or CD8-positive cells from normal donors were used to compare the replication kinetics of AD3.v6 and AD3.v22 viruses.

Two million PHA-activated (1 μg/ml) PBL or purified CD4-positive and CD8-positive cells were infected with the HIV-1 isolates for 2 hours at 37°C using 0.05 pg of p24 per cell. Cells were washed, adjusted to 10^6 cells/ml and cultured in complete RPMI 1640 medium supplemented with human recombinant IL-2 (50 units/ml) (Life Technologies, Grand Island, NY). Culture supernatants were harvested at regular intervals and stocked at –80° C until assayed for p24 antigen using a kit (Coulter, Hialeah, FL). Phenotyping was performed by FACS at regular intervals using directly labeled anti-CD4 (clone RPA-T4, Zymed Laboratories, San Francisco, CA), anti-CD8 (Sigma Chemicals, St. Louis, MO) or indirectly labeled anti-CCR5 2D7, Pharmacia International, San Diego, CA) and anti-CXCR4 (12G5, J. Hoxie, AIDS Research and Reference Reagent Program) antibodies with appropriate isotype and cell controls.

AD3.v6 and AD3.v22 efficiently replicated in PBL and purified CD4-positive cells. Surprisingly, in repeated experiments, these viruses were also able to replicate in CD8-positive cells at a level that matched the level in PBL and purified CD4-positive cells indicating that CD8-positive lymphocytes are targets for these viruses. CD4-tropic HIV-1/IIIB (AIDS Research and Reagent Program, NIH) was used as a control and primarily replicated in PBL and CD4-positive cells but not in CD8-positive cells.

Several lines of evidence suggest that CD4 played no role in production of AD3.v6 and AD3.v22 by the purified CD8-positive population of cells. First, the phenotypes of purified cells were measured all through the experiments. Only a small number of cells in the CD8-positive population were CD4-positive before infection (0 day), at the time of peak virus production (18 day) or at any point of time in between. Second, compared to purified CD8-positive cells, PBL contained a much larger number of CD4-positive cells. Had CD4-positive cells been the only targets for these viruses, virus production would have been higher in PBL compared to CD8-positive cells. Third, although these viruses were able to replicate in CD4-positive cells, virus production in CD8-positive cells was comparable to that in CD4-positive cells indicating that CD8-positive cells are an equally good target for AD3.v6 and AD3.v22 as CD4-positive cells.

Further evidence that contaminating CD4-positive cells did not produce viruses in purified CD8-positive cells comes from triple-color FACS analyses. Triple-color FACS analyses were performed using a kit from Coulter by fixing the cells first, followed by staining with FITC-p24, CD8-PE and CD4-PC5. At 8 days post infection (8 dpi) when for the first time virus-expressing cells could be detected, most (72.1%) virus-expressing cells were CD8-positive/CD4-negative. The number of CD8-positive/CD4-negative cells expressing HIV-1 increased with progression of infection, and around the time of peak virus production (19 dpi) all HIV-expressing cells were CD8-positive/CD4-negative. Also, in a control experiment where both CD4-positive and CD8-positive cells were removed from PBL and residual (non-CD4/CD8) cells were infected with AD3.v6 or AD3.v22, little or no virus production was detected indicating that any CD8-negative/CD4-negative cells contaminating the CD8-positive population were not be the targets for these viruses

Interestingly, expression of CD8 was down-modulated after infection of CD8-positive cells with AD3.v6 or AD3.v22. CD4 was also down-modulated after infection of CD4-positive cells with these viruses suggesting maintenance of CD4-tropism.

In another type of experiment, purified CD8-positive cells were sorted shortly after infection with AD3.v6 or AD3.v22 and these stringently selected CD8-positive

cells were tested for expression of CD4 and HIV-1 by RT-PCR to rule out the possibility that the CD8-positive cells were expressing too low a level of CD4 to be detected by FACS.

Briefly, purified CD8-positive cells were FACS sorted after staining with CD8-PE and CD4-PC5 antibodies using a gate to isolate CD8-positive/CD4-negative and CD8-negative/CD4-negative populations. Sorted cells were washed several times and either tested immediately for expression of CD4, CD8 or HIV-1 mRNA by RT-PCR. Total RNA was extracted using Rneasy Mini Kit (Qiagen, Valencia, CA). DNA-free RNA was reverse transcribed using Qiagen Omniscript RT system and tested for expression of CD4, CD8, or HIV-1 using specific primers.

AD3.v6- and AD3.v22-infected and sorted CD8-positive cells expressed CD8 as well as HIV-1 (*gag*) but no CD4, while uninfected CD8-positive cells expressed CD8 but no CD4 or HIV-1. Similarly sorted CD8-positive cells after infection with CD4-tropic (JR-FL) viruses did not express HIV-1 or CD4. Thus, it is clear that sorted CD8-positive cells were free from CD4-positive cell contamination.

Taken together, these results demonstrate that AD3.v6 and AD3.v22 are able to target CD8-positive/CD4-negative cells and that CD4 played no role in infection of the cells.

Example 2

The foregoing experiments with primary CD8-positive cells strongly support that CD8-positive cells are targets for AD3.v6 and AD3.v22. However, since primary cells contain a mixture of different cell types as discussed above, to further establish CD8-tropism of these viruses, additional experiments were performed using a CD8-positive, CD4-negative T-cell line, KRCD8, described in Saha *et al.*, *J. Virol.* *72*:876-881 (1998).

KRCD8 were infected as described in Example 1 with AD3.v6, AD3.v22 and HIV-1/IIIB and p24 production was measured at regular intervals. AD3.v6 and AD3.v22, but not HIV-1/IIIB, were able to infect KRCD8 cells. No expression of CD4 was detected in KRCD8 cells whether uninfected or after infection, even by

sensitive RT-PCR. These results demonstrate that KRCD8 cells were infected by AD3.v6 and AD3.v22 through a CD4-independent mechanism.

As recent reports of CD4-independent infection with HIV (see articles discussed in the Background) have implicated CCR5 or CXCR4 as the alternative pathway to infection using CD4, the KRCD8 cells were also examined for expression of those coreceptors. Uninfected KRCD8 cells did not express CCR5 or CXCR4 nor did infected KRCD8 cells, indicating that infection of KRCD8 cells was independent of CCR5 or CXCR4.

These studies thus confirm that AD3.v6 and AD3.v22 infect CD8-positive cells in a CD4-independent manner.

Since KRCD8 cells were susceptible to AD3.v6 and AD3.v22, it was investigated whether these viruses used CD8 receptors to infect these lymphoid cells. AD3.v6, AD3.v22 or HIV-1/IIIB viruses were used to infect KRCD8 cells or MT-2 cells (control) in the presence of anti-CD8 (C1) (clone SPV-T8, Zymed Laboratories, San Francisco, CA), -CD4 (clone RPA-T4, Zymed Laboratories) or isotype control antibodies. While isotype control or anti-CD4 antibodies had no effect on infection by AD3.v6 or AD3.v22, anti-CD8 antibodies (C1) blocked entry of these viruses into KRCD8 cells. However, anti-CD8 antibodies were not able to prevent infection of CD4-positive MT-2 cells by AD3.v6, AD3.v22 or HIV-1/IIIB viruses. In contrast, anti-CD4 antibodies blocked infection of MT-2 cells by AD3.v6, AD3.v22 and HIV-1/III viruses.

Taken together, these results confirm that AD3.v6 and AD3.v22 use CD8 receptors for infection of CD8-positive cells. The results also demonstrate that these viruses used CD4 receptors to infect CD4-positive cells.

Example 3

The ability of AD3.v6 and AD3.v22 to infect cell lines transfected to express CD8 was examined.

First, HeLa T8+ cells, a CD8-transfected tumor cell line that does not express CD4 were examined. HeLa T8+, HeLa T4+ (susceptible to CD4-tropic HIV-1) or parental HeLa cells were infected with AD3.v6, AD3.v22 or HIV-1/III.

Infections of HeLa T8+, HeLa T4+ or parental HeLa cells were performed either by co-culturing cells with infected PBL or with cell-free viruses in the presence of polybrene (2 μg/ml). After overnight co-culture or infection for 4 hours with cell-free viruses, cells were thoroughly washed, trypsinized and re-plated with fresh medium. Every 5-7 days intervals, cells were trypsinized and re-plated. Culture supernatants were harvested at regular intervals and assayed for p24. For non-productive infections, infection was detected at routine intervals by PCR.

Viral replication was observed between 7-15 days after infection with AD3.v6 and AD3.v22, but not with HIV-1/III. However, all three viruses were able to infect HeLa T4+, but not parental HeLa cells. Like KRCD8 cells, infection of HeLa T8+ cells with AD3.v6 and AD3.v22 was independent of CD4 expression as monitored through RT-PCR. Expression of CD8 was also down-modulated in HeLa T8+ cells after infection with AD3.v6 and AD3.v22 further indicating the role of CD8 as a receptor for those viruses.

The HeLa T8+ cells were also infected with AD3.v6, AD3.v22 or HIV-1/III in the presence of anti-CD8 (C1), anti-CD4 or isotype control antibodies described above. AD3.v6 and AD3.v22 were able to infect HeLa T8+ and HeLa T4+ cells, but not HeLa cells and viral entry into HeLa T8+ cells was blocked with anti-CD8, but not with isotype control or anti-CD4 antibodies. In contrast, viral entry into HeLa T4+ cells was prevented by anti-CD4 antibodies, but not with isotype control or anti-CD8 antibodies. As expected, HIV-1/III viruses were only able to infect HeLa T4+, but not HeLa T8+ or HeLa cells.

Next, to further test the role of CD8 as a receptor for AD3.v6 and AD3.v22, a monkey kidney cell line (COS-T8) that constitutively expressed high levels of human CD8 was generated.

To generate human CD8-expressing COS cells, T8pMV7 vector (AIDS Research & Reagent Program) expressing CD8 was used to transfect COS-7L cells (GIBCO-BRL). Lipofectamine 2000 (GIBCO-BRL), a lipid transfecting reagent was used for transfection according to the manufacturer's instruction. Twenty-four hours post-transfection, cells were re-plated at 1:20 dilution and after another 24h, cells

were put in selection medium. Individual clones were isolated and screened by FACS to select clones expressing CD8.

Like HeLa T8+ cells, AD3.v6 and AD3.v22 viruses were able to infect COS-T8, but not parental COS cells while HIV-1/III viruses failed to infect either of these cell lines. No CD4 expression was detected in COS-T8 cells before or after infection.

These results establish that AD3.v6 and AD3.v22 are able to infect both types of transfected cells using CD8 as a receptor.

Example 4

The ability of anti-CD8 antibodies to inhibit replication of AD3.v6 and AD3.v22 in primary CD8-positive cells was tested.

For inhibition of viral replication, specific monoclonal antibodies or isotype control antibodies (5 μg/ml) were added to purified CD8-positive cells for 30 minutes at room temperature prior to infection. After infection for one hour, cells were washed and re-suspended in culture medium with AD3.v6 or AD3.v22 virus to which respective monoclonal antibodies were added. Culture supernatants were harvested at regular intervals and assayed for p24. Half of the medium was replaced twice every week with fresh medium containing respective antibodies except in some experiments where antibodies were stopped after two weeks.

Replication of AD3.v6 and AD3.v22 viruses was significantly inhibited by anti-CD8 (C1) antibodies, but not by isotype control antibodies. Anti-CD8 antibodies had little effect on replication of these as well as HIV-1/III viruses in CD4-positive cells. As discussed above, AD3.v6 and AD3.v22 have maintained an unchanged ability to use CD4 receptors and should be considered dual (CD4/CD8)-tropic. Since CD4 is co-expressed in some CD8-positive cells, it is conceivable that these viruses may use CD8 as well as CD4 receptors to infect double-positive cells.

Example 5

Cellular tropism of HIV-1 is primarily determined by viral envelope polypeptides, in particular gp120. In order to further characterize CD8-tropic HIV-1, DNA encoding gp120 envelopes of AD3.v6 and AD3.v22 was sequenced. Full length

envelope coding regions were amplified by nested PCR from genomic DNA using outer (5'-CTGGAAGCATCCAGGAAGTCAGCC-3; SEQ ID NO: 23 and 5'-GTCCCCAGCGGAAAGTCCCTTGTA-3'; SEQ ID NO: 24) and inner (5'-GAGACAGTGGCAATGAGAGTGAAGG-3'; SEQ ID NO: 25 and 5'-CTTTTTGACCACTTGCCACCCATCTT-3': SEQ ID NO: 26) primers. Amplified PCR fragment (2.6 kb) was purified and sequenced from both DNA strands by cycle sequencing on an ABI 377 DNA Sequences. Sequence assembly and comparisons were performed with Lasergene (DnaStar, Madison, WI) as well as with NCBI Blast Server. The DNA sequence encoding AD3.v6 gp120 envelope polypeptide is set out in Fig. 1, while the DNA sequence of AD3.v22 gp120 is set out in Fig. 2.

An alignment of the protein sequences encoded by the DNAs with the gp120 amino acid sequence of HXB2, a prototype CD4-tropic isolate, and the closest published gp120 amino acid sequence, WEAU1.6, is presented in Figure 4. In the figure, dashes signify 100% identity among sequences and dots signify the absence/deletion of a base. Differences between AD3.v6/AD3.v22 viruses and WEAU1.6 are colored red in the WEAU1.6 sequence. Figure 4 displays the gp120 polypeptide and clearly indicates the location of the V1, V2, V3, V4 and V5 loops.

Extensive differences exist throughout AD3.v6 and AD3.v22 gp120 when compared with HXB2 and with WEAU1.6. The changes were most extensive in variable (V) loops and a striking feature of these changes was an extended V1-V2 loop. Extensive differences were also present in the important V3 loop when compared to its closest match. In contrast, only a single point change was observed in the entire gp41 between AD3.v6 and AD3.v22 viruses (not shown). Interestingly, the CD4 binding region (in blue) and other residues that are known to be important for CD4-tropism (underlined) remained unchanged.

Although no sequence has been definitely been correlated with a specific HIV-1 phenotype, critical residues in gp120 have been identified that are important for cellular tropism through CCR5 or CXCR4 rather than CD4. For example, it has been recently demonstrated that with an IGX motif at positions 348-350 in Fig. 4 in the V3 loop and with a basic residue at position 331, viruses are incapable of using CCR5. These residues (boxed) were identical among AD3.v6, AD3.v22 and HXB2 viruses

which cannot use CCR5, suggesting that AD3.v6 and AD3.v22 viruses probably do not use CCR5 co-receptors. Also, CD4-independent infection by viruses using CCR5 has been correlated with specific gp120 changes (italics) resulting in loss of N-linked glycosylation sites at distinct regions. Although extensive changes in V1-V2 loop of AD3.v6 and AD3.v22 viruses were observed, these viruses maintained these specific glycosylation sites (marked with • in Fig. 4). CD4-independent entry through enhanced use of CXCR4 has also been correlated with seven specific mutations (bold). A specific T to S substitution, not present in either AD3.v6 or AD3.v22 , was necessary for CD4-independent infection.

Thus, although AD3.v6 and AD3.v22 viruses use CXCR4 coreceptors for infection of CD4-positive cells (data not shown), CXCR4 and CCR5 apparently are not necessary for infection through CD8 receptors.

Example 6

To identify additional CD8-tropic HIV-1, viral stocks from twelve patients were used to test for the presence of CD8-tropic virus. It is contemplated that CD8-tropic HIV quasispecies may be present in a patient at any given time after infection. Although these CD8-tropic viruses exist in an infected patient, these viruses probably are at least initially, outnumbered by the more common CD4-tropic viruses.

Therefore, in order to isolate CD8-tropic viruses from the quasispecies, CD8-tropic virus may be enriched from the original viral stocks. In Example 1, the CD8-tropic HIV-1 viruses AD3.v6 and AD3.v22 were isolated from Herpes virus saimirir (HVS)-transformed CD8-positive T cell clones from a single patient. The additional CD8-tropic HIV-1 viruses described herein (92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8) were isolated without using HVS by carefully avoiding CD4-tropic contaminants. These procedures eliminated the remote possibility of HVS influencing HIV-tropism.

Twelve primary HIV-1 isolates of different clades (obtained through AIDS Res. & Ref. Program, NIH) were randomly selected. The viruses were originally isolated from different HIV-1-infected patients in North America, Africa and Asia (Table 2). As listed by the AIDS Res. & Ref. Program, these viruses contained

CCR5-, CXCR4- or dual (CXCR4/CCR5)-tropic isolates of different clades. In Table 2 these receptors are denoted as R5 (CCR5), X4 (CXCR4) or R5X4 (CXCR4/CCR5).

The approach used to isolate CD8-tropic HIV-1 viruses from infected CD8-positive cells was previously described in Saha *et al. Nature Medicine*, 7: 65-72 (2001). First, purified (CD4-depleted) CD8-positive cells from normal donors were infected with viral stocks from different patients. Three to five days after infection, infected CD8-positive cells were further sorted from contaminant CD4-positive cells by FACS analysis using antibodies against different T cell surface markers including CD4 and CD8, and a very conservative gate to select unadulterated CD8-positive/CD4-negative populations. This analysis reduced the chance of including CD4-tropic isolates that may have been carried through contaminating CD4-positive cells or through the infection of double-positive (CD8-positive/CD4-positive) cells. Finally, CD8-tropic viruses were grown from infected CD8-positive cells by co-culture with CD4-negative depleted cells from normal donors using 0.05 pg of p24/cell.

The resulting infected and sorted CD8-positive cells were tested for the presence of CD4 mRNA and HIV-1 by RT-PCR and PCR. The cells which expressed any CD4 mRNA were discarded from further experiments to avoid any possibility for inclusion of CD4-tropic contaminants. Finally, CD8-tropic viruses were isolated by co-culturing infected (HIV-1 DNA-positive) CD8-positive cells with purified CD8-positive cells from normal donors.

All sorted CD8-positive cells were free from CD4 contamination. At the time of sorting, little or no virus production was detected with most of the infected CD8-positive cell cultures ruling out the possibility of virus-induced down-modulation of CD4 molecules. HIV-1 DNA (gag) was detected in CD8-positive cells from seven out of the twelve patients indicating the possible presence of CD8-tropic isolates in these individuals (Table 2).

Table 2

Patient	Clade	Country	Sex/Age	Disease	CD4-tropism (co-receptor)	HIV-DNA in CD8+ cells
92UG046	D	Uganda	M/25	Asymptomatic	SI, X4	+
93UG086	D	Uganda			SI, R5X4	8
92US077	B	USA	Infant		R5X4	+
93US143	B	USA	Infant		SI, R5X4	+
96USHIPS4	B	USA	F/teen-age	AIDS	R5X4	+
96USHIPS9	B	USA		AIDS	R5X4	+
96USSN20	A	USA/ Senegal	M	AIDS	R2B,3,4,5,X4	+
92UG001	D	Uganda	M/26	Asymptomatic	R5X4	-
92US727	B	USA			R5	-
91US056	B	USA	Infant		R5	-
93US151	B	USA	Infant		R5X4	-
CMU08	E	Thailand			X4	-

Viruses from all twelve patients replicated, albeit at different levels, in CD4-positive cells. HIV-1 viruses from the patients that did not infect CD8-positive cells (*e.g.* 92UG001, 92US727, 91US056) replicated to a much higher level in CD4-positive cells compared to some of the viruses that were able to infect CD8-positive cells. This suggests that infection of CD8-positive cells is not due to the presence of contaminating CD4-tropic isolates.

Virus production was detected, albeit at lower levels when compared to CD4-positive cells, from CD8-positive cells infected with viruses from five out of the seven patients which tested positive for HIV-1 DNA in CD8-positive cells. The reason for the lack of virus production by CD8-positive cells in spite of being positive for HIV-1 DNA from two patients (96UG046 and 96USHPS9) is not clear. It is possible that viruses from these two patients replicated poorly in CD8-positive cells. However, as described later (see Example 7), viruses from these two patients did replicated in co-culture with CD8-positive cells. These results indicate the presence of CD8-tropic viruses in some of the patients that induced infection in CD8-positive cells, albeit at lower levels compared to CD4-positive cells.

Example 7

The foregoing experiments demonstrate the existence of CD8-tropic viruses in various HIV-1 patients. The following studies were carried out to further characterize and substantiate the CD8-tropism of viruses 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8. For growth of HIV-1 virus *in vitro*, short-term co-culture with the target cells from normal donors was necessary. In order to grow CD8-tropic viruses, infected CD8-positive cells from all patients (PCR-positive or -negative for HIV-1 DNA) were co-cultured with purified CD8-positive or CD4-positive cells from normal donors.

As summarized in Table 3 below, production was readily detected when viruses 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8 were co-cultured with CD4-positive or CD8-positive cells. With most of the isolates, the levels of virus production were higher in the presence of CD4-positive cells when compared to CD8-positive cells suggesting that like the CD8-tropic viruses AD3.v6 and AD3.v22 (Example 1), the CD8-tropic viruses 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8 are also dual (CD4/CD8)-tropic and probably replicate more efficiently in CD4-positive cells.

Table 3

Co-Cultured with CD8-Positive Cells

HIV-1/T8 Viruses	RT x1000 (cpm/ml)	Viral load* (copies/ml) x10^5	$TCID_{50}$/0.2ml†
1. 92UG046-T8	27	<50	10^8
2. 93UG086-T8	454	52.1	$>10^7$
3. 92US077-T8	632	>37500	$10^{6.85}$
4. 93US143-T8	551	7229.25	$10^{8.3}$
5. 96USHIPS4-T8	1780	2604	10^7
6. 96USHIPS9-T8	72	<50	$10^{6.5}$
7. 96USSN20-T8	2722	5.3	$10^{6.5}$
8. AD3.v6	3914	4870.3	$10^{5.75}$

Co-Cultured with CD4-Positive Cells

HIV-1/T8 Viruses	RT x1000 (cpm/ml)	Viral load (copies/ml) x10^5	$TCID_{50}$/0.2ml
1. 92UG046-T8	45	<50	$10^{6.9}$
2. 93UG086-T8	2420	176.8	$>10^7$
3. 92US077-T8	246	946.6	10^7
4. 93US143-T8	678	29250	$>10^7$
5. 96USHIPS4-T8	3097	1298	$>10^7$
6. 96USHIPS9-T8	624	5092.2	10^7
7. 96USSN20-T8	2466	6.4	$>10^7$
8. AD3.v6	ND¶	1764.5	ND

*Viral load was detected using AMPLICOR kit.
†$TCID_{50}$ was measured with MT-2 cells.
¶ND stand for not done

Example 8

The CD8-tropic viruses 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8 were able to infect the CD8-positive/CD4-negative cell line, KRCD8 as shown by experiments carried out as described in Example 2. In the experiments, all of the CD8-tropic viruses had the ability to infect KRCD8 cells as indicated by the presence of viral DNA soon after infection and by induction of HIV-specific transcripts. Virus from patients that did not exhibit CD8-tropism 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8 were unable to replicate in KRCD8 cells.

Infection of KRCD8 cells by the CD8-tropic viruses was also confirmed by detection of viral particles using electron microscopy and by the ability of KRCD8 cell-produced viruses to transactivate β-galactosidase expression under the control of the HIV-LTR promoter. The transactivation assays were carried out using MAGI cells as described in Huang *et al. J. Virol.* 72: -2047-2054, (1998).

Finally, experiments testing the ability of anti-CD4 antibodies to block infection of KRCD8 cells by CD8-tropic viruses 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8 were carried out as described in Example 3. Infection of KRCD8 cells by the CD8-tropic viruses could not be blocked by the addition of anti-CD4 antibodies that were able to block infection of CD4-positive cells by CD4-tropic HIV-1.

Together, these data establish that the CD8-tropic viruses 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8 are able to infect CD8-positive cells independent of CD4 as are AD3.v6 and AD3.v22.

Example 9

In another experiment, the ability of the CD8-tropic viruses to induce syncitia in CD8-positive cells was investigated. CD4-tropic viruses are classified as SI or NSI based on their ability to induce or not to induce syncitia in CD4-positive cells, respectively. Induction of syncitia in CD4-positive cells by CD4-tropic viruses is generally mediated through the use of CXCR4 co-receptors. All CD8-tropic viruses in this study were also of SI phenotype in primary CD4-positve cells (Table 4) as well as in MT-2 (CD4-positive) cells.

Table 4

HIV-1/T8 Viruses	Syncitia KDC8 cells	Syncitia MT2 cells	Syncitia (co-culture with CD8-Positive Cells)	Syncitia (co-culture with CD4-Positive Cells)
1. 92UG046-T8	+	+	+	+
2. 93UG086-T8	-	+	-	+
3. 92US077-T8	-	+	-	+
4. 93US143-T8	+	+	+	+
5. 96USHIPS4-T8	-	+	-	+
6. 96USHIPS9-T8	+	+	+	+
7. 96USSN20-T8	-	+	-	+
8. AD3.v6	-	+	-	+

CD8-tropic viruses from three patients (92UG046-T8, 93US143-T8 and 96USHIPS9-T8) were also able to induce syncitia in primary CD8-positive cells (Table 4). These three CD8-tropic viruses also induced striking syncitia 2-5 days after infection in KRCD8 cells. Induction of syncitia in CD8-positive cells by the three CD8-tropic viruses was quickly followed by extensive cell death. As shown above, although CD8-tropic viruses 93UG086-T8, 92US077-T8, 96USHIPS4-T8, and 96USSN20-T8 from the other five patients were able to infect KRCD8 cells, none of the viruses were able to immediately induce syncitia in the KRCD8 cells.

Taken together, these results demonstrate that CD8-tropic viruses may also be of SI or NSI phenotypes in CD8-positive cells

Example 10

The DNA sequences of the envelope proteins of the CD8-tropic viruses 92UG046-T8, 93UG086-T8, 92US077-T8, 93US143-T8, 96USHIPS4-T8, 96USHIPS9-T8 and 96USSN20-T8 were determined and the sequences are set out as SEQ ID NOS: 10, 12, 14, 16, 18, 20 and 22.

The full length envelope coding regions of the CD8-tropic viruses were amplified by nested PCR from infected CD8-positive cells and the amplified PCR fragments were sequenced from both DNA strands using a panel of primers (SEQ ID NOS: 16-19) by cycle sequencing on an ABI 377 DNA sequencer as previously described in Example 5. Envelope sequences from CD4-positive cells infected with

the bulk viral stocks from these patients were also examined and compared to the CD8-tropic envelope viral sequences. Other envelope sequences from HIV-1 viruses isolated from these patients are available in the Los Alamos HIV database from previous studies and were compared with the isolated CD8-tropic sequences of the invention. Sequence assembly and comparisons were performed with Lasergene (DnaStar, Madison, WI) as well as with NCBI Blast server. The envelope sequences of the CD8-tropic viruses have been submitted to Genbank (accession numbers AF 391548 - 391553).

A comparison of the envelope sequences from a representative CD8-tropic virus 92UG046-T8 with the previously published sequences (obtained from infected CD4-positive cells) is shown in Figure 6. In the figure, dashes signify 100% identity among sequences and dots signify the absence/deletion of a base. Figure 6 displays the full length envelope polypeptide sequence (gp120 and gp41). Locations of the V1, V2, V3, V4 and V5 loops are clearly identified in Figure 6 along with the location of where the gp120 polypeptide ends. Several amino acid changes were observed between the envelopes of isolated CD8-tropic viruses when compared to the sequences in the Los Alamos database. These changes were scattered in the important V1-V2, C2 and V4 loops. Other CD8-tropic viruses also had diverse changes in the variable as well as transmembrane regions. The CD8-tropic AD3.v6 and AD3.v232 identified in Example 1, also had interesting changes in the V1, V2 and C2 regions.

Sequence analysis (VESPA) identified particular changes which are associated with CD8-tropism. The VESPA analysis was carried out with a threshold at 80% to compare the frequencies of residues at a specific site as compared to a background set (Los Alamos HIV database). The sites associated with CD8-tropism are contemplated to be those at which the frequency in the query set is greater than the frequency in the background set. These changes are summarized in below in Table 5.

Table 5

Position	Protein Domain	Query residue	Frequency*	Background residue	Frequency
270	C2	I	80%	V	85.1%
693	gp41	I	100%*	V	58.8%
724	gp41	Q	80%	P	63.8%
779	gp41	A	80%	T	58.5%
177	V2	D/E	88.3%*	Q	60%
185	V2	D/E	83%*	K/R	40%
187a	V2	gap	81.9%*	N	40%
187b	V2	gap	81.9%*	N	60%
187c	V2	gap	81.9%*	N	60%
209	C2	S	98.9%*	T	40%
352	C3	Q	89.4%*	H	60%
400a	V4	gap	98.9%*	D/E	40%
442	C4	Q	81.9%*	L	40%
662	gp41	D/E	85.1%*	A	60%

*Significant Frequency

No sequence change was observed among the CD8-tropic viruses in their CD4-binding region (bold in Fig. 6) or other residues known to be important for CD4-tropism indicating that mutations in the CD8-tropic strains are probably not just random events.

Additional sequence analysis revealed the CD8-tropic envelope sequence from the 92UG046-T8 isolate a stop codon within the transmembrane domain which resulted in a truncated gp41 proteins with a cytoplasmic tail about 70 amino acids shorter than the CD4-tropic envelope protein from the same patient. In addition, the CD8-tropic env sequence from the 93UG086-T8 and 96USSN20-T8 viruse both have a seven amino acid insert (HSSLKGL; SEQ ID NO: 27) towards the end of the transmembrane domain.

Example 11

The foregoing examples demonstrate for the first time that HIV-1 can mutate to a form that can infect CD8-positive cells using CD8 as a primary receptor. These data are significant because despite a strong CD8-positive cell-mediated immune response after primary HIV infection, the host immune defense eventually fails leading to the development of AIDS. Although several hypotheses including anergy, apoptosis and antigenic stimulation have been put forward, the exact reason for ultimate failure of CD8-positive cells had been unclear. The existence of CD8-tropic HIV-1 can explain the failure of CD8-positive cells in AIDS patients. With increasing selective pressure from a declining pool of CD4-positive cells as infection progresses, HIV-1 evolves to be able to infect CD8-positive cells. Productive infection of immune-competent CD8-positive cells with CD8-tropic HIV-1 results in functional defects in these cells or kills these cells leading to a quantitative failure of the immune system and progression to AIDS. Blocking infection of CD8-positive cells by CD8-tropic HIV-1 is thus indicated as a vaccination strategy against, and as a therapy for, HIV-1 infection.

While the present invention has been described in terms of preferred embodiments, it is understood that variations and improvements will occur to those skilled in the art. Therefore, only such limitations as appear in the claims should be placed on the invention.